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Enhanced Copper Tolerance in *Silene vulgaris* (Moench) Garcke Populations from Copper Mines Is Associated with Increased Transcript Levels of a 2b-Type Metallothionein Gene[1]

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Silene vulgaris (Moench) Garcke has evolved populations with extremely high levels of copper tolerance. To evaluate the role of metallothioneins (MTs) in copper tolerance in *S. vulgaris,* we screened a cDNA library derived from a highly copper-tolerant population using Arabidopsis-based MT probes and identified an MT2b-like gene. When expressed in yeast, this gene, *SvMT2b*, restored cadmium and copper tolerance in different hypersensitive strains. Northern-blot analysis and quantitative reverse transcriptase-PCR showed that plants from the copper-tolerant *S. vulgaris* populations had significantly higher transcript levels of *SvMT2b* than plants from the copper-sensitive populations, both in roots and shoots and with and without copper exposure. Southern-blot analysis suggested that the higher expression of the latter allele was caused by gene amplification. Segregating families of crosses between copper-sensitive and copper-tolerant plants exhibited a 1 to 3 segregation for *SvMT2b* expression. Allele-specific PCR showed that low-expression F_3 plants were homozygous for the allele inherited from the copper-sensitive parent, whereas high-expression plants possessed at least one allele from the tolerant parent. *SvMT2b* expression did not cosegregate with copper tolerance in crosses between sensitive and tolerant plants. However, a significant cosegregation with copper tolerance did occur in families derived from crosses between moderately tolerant F_3 plants with different *SvMT2b* genotypes. Thus, overexpression of *SvMT2b* conferred copper tolerance although only within the genetic background of a copper tolerant plant.

The Bladder Campion, *Silene vulgaris* (Moench) Garcke, has evolved strongly heavy metal-tolerant populations at sites with high heavy metal concentrations in the soil (Ernst, 1974; Schat et al., 1996). The mechanisms underlying such high-level tolerance are largely metal specific (Schat and Vooijs, 1997). High-level copper tolerance in *S. vulgaris*, as suggested by the segregation patterns in crosses between plants from copper-sensitive and copper-tolerant populations, may be controlled by two primary tolerance genes (major genes) and some hypostatic "modifiers" (Schat and Ten Bookum, 1992b; Schat et al., 1993, 1996). The nature and physiological functions of these genes have not been identified yet.

Metallothioneins (MTs) might be involved in copper tolerance. MTs are low molecular weight, Cys-rich cytoplasmic metal-binding proteins that could protect cells against the toxic effects of copper by chelating this heavy metal. Genes encoding MTs occur in animals, higher plants, eukaryotic microorganisms, and in some prokaryotes. There are different MT families, subfamilies, subgroups, and isoforms. A review of MTs in plants and their characteristics is given by Rauser (1999). Several plant species contain large MT gene families, consisting of different MT gene types, and/or multiple MT genes of one type (Zhou and Goldsbrough, 1995; Hudspeth et al., 1996). The similarity between MT genes within one species is often very high. Moreover, three MT genes are situated within 20 kb of the same chromosome in cotton (Hudspeth et al., 1996). Likewise, two type 1 MT genes are found within 3 kb in Arabidopsis (Zhou and Goldsbrough, 1995). These findings strongly suggest that gene amplification is involved in the evolution of MT genes.

The functions of MTs in plants are still unclear (Rauser, 1999). However, Murphy and Taiz (1995) found that *MT2* expression was the primary determinant of ecotypic differences in the copper tolerance of Arabidopsis seedlings. Moreover, heavy metal tolerance in plants can be improved by (over) expression of yeast MT genes (Hasegawa et al., 1997), and plant MT genes can restore metal tolerance in MT-deficient yeast (Zhou and Goldsbrough, 1994). For these rea-

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sons, it is conceivable that MTs might play a role in copper tolerance in *S. vulgaris*.

This study was performed to isolate and characterize MT genes from *S. vulgaris*, and to investigate their role in copper tolerance in different populations of this species. The latter was done by heterologous expression in hypersensitive yeast, as well as by analyzing the co-segregation of MT expression and copper tolerance in segregating families of crosses between copper-sensitive and -tolerant plants.

RESULTS

MT Sequences

A cDNA library was prepared from leaves of untreated copper-tolerant plants (population Imsbach). Of the Arabidopsis-based MT probes tested (*MT1a*, *MT2a*, *MT2b*, *MT3*), only *MT2b* yielded hybridizing plaques. The corresponding *S. vulgaris* cDNA (*SvMT2b*; GenBank accession no. AF101825) showed a high amino acid sequence identity with Arabidopsis MT2b (Zhou and Goldsbrough, 1995; GenBank Accession no. U11256) and *Mesembryanthemum crystallinum MT* (GenBank accession no. AF000935), particularly in the Cys-rich N-terminal and C-terminal parts (Fig. 1). The *SvMT2b* gene sequences were also determined in two other populations of *S. vulgaris*. The *SvMT2b* cDNA sequence of plants from Amsterdam differed at 7-bp positions from the Imsbach cDNA. These resulted in three amino acid substitutions in the spacer region (Ser for Asn, Gly for Ala, and Met for Lys, at the positions 18, 32, and 46 of the predicted protein). The cDNA of plants from Marsberg differed at 5-bp positions from the Imsbach cDNA, resulting in one amino acid substitution (Met for Lys at position 46).

Yeast Complementation

A copper-sensitive yeast, DBY746, transformed with *SvMT2b*-pAJ401 was able to grow at 5 mM $CuSO_4$, whereas the untransformed DBY746 mutant grew up to 1 mM copper. Copper tolerance of the copper-sensitive yeast was thus approximately 5-fold increased. The MT-deficient copper-sensitive yeast *cup1* mutant DM771–6C transformed with *SvMT2b* grew at 1 mM $CuSO_4$, but the untransformed mutant did not grow at 0.5 mM copper.

A cadmium-sensitive yeast, JWY53 (vacuolar membrane ABC transporter mutant *ycf1*), transformed with *SvMT2b*-pAJ401 was able to grow at 0.1 mM $CdSO_4$, whereas the original yeast mutant with or without pAJ401 plasmid grew up to 0.01 mM $CdSO_4$ (Fig. 2).

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Sv  MSCCNGNCGC  GSACKCGSGC  GGCKMFPDFA  E-GSSGSASL  VLGVAPMASY  49
At  MSCCGGSCGC  GSACKCGNGC  GGCKRYPDLE  N---TATETL  VLGVAPAMNS  47
Mc  MSCCGGNCGC  GSACKCGNGC  GGCKMYPDMA  ENGASSTATL  VTGVAPKISY  50

Sv  FDAEMEMGVA  TEN-GCKCGD  NCQCNPCTCK  78
At  QYEASGETFV  AENDACKCGS  DCKCNPCTCK  77
Mc  FDNGSEMGVG  AENDGCKCGS  DCKCDPCTCK  80
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Figure 1. Alignment of amino acid sequences of *S. vulgaris* SvMT2b (population Imsbach; *Sv*), Arabidopsis MT2b (*At*), and *M. crystallinum* MT (*Mc*).

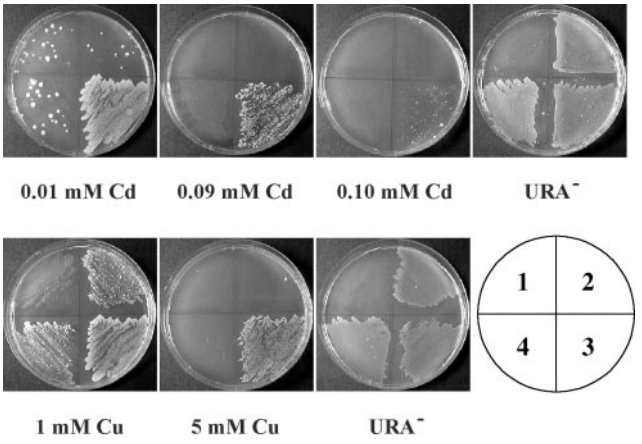

Figure 2. Expression of *SvMT2b* in yeast. Yeast strains JWY53 (Δ*ycf1*) (upper) and DBY746 (lower) growing on PDA plates supplemented with cadmium or copper as sulfates: 1, untransformed; 2 and 4, transformed with empty vector (pAJ401); and 3, transformed with *SvMT2b*.

SvMT2b mRNA Expression Analysis in Parental Ecotypes

Both northern-blot hybridization and quantitative RT-PCR showed much higher levels of *SvMT2b* mRNA in the roots (Fig. 3) and leaves (data not shown) of the plants from the copper-tolerant populations Imsbach and Marsberg than in those from three copper-sensitive populations Amsterdam (Fig. 3), Wijlre and Gaschurn (data not shown). *SvMT2b*

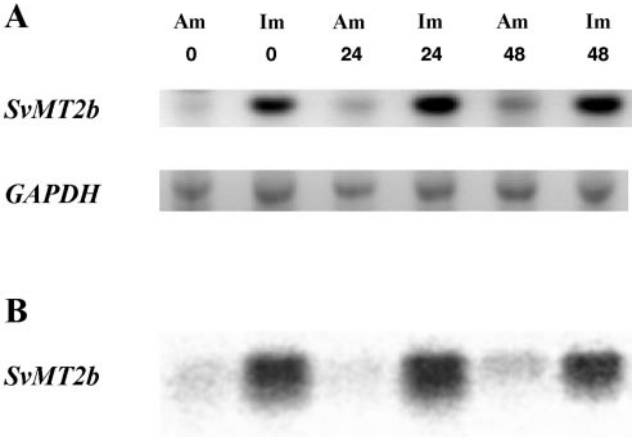

Figure 3. SvMT2b expression in roots of copper-sensitive (Am, Amsterdam) and copper-tolerant plants (Im, Imsbach), unexposed (0) or exposed to 50 μM $CuSO_4$ for 24 and 48 h (respectively, 24 and 48). A, Ethidium bromide-stained agarose gel of the quantitative RT-PCR products. Also included is the internal control, *GAPDH*. B, Northern-blot analysis. Each lane was loaded with 8 μg of total RNA. Radioactively labeled *SvMT2b* was used as a probe.

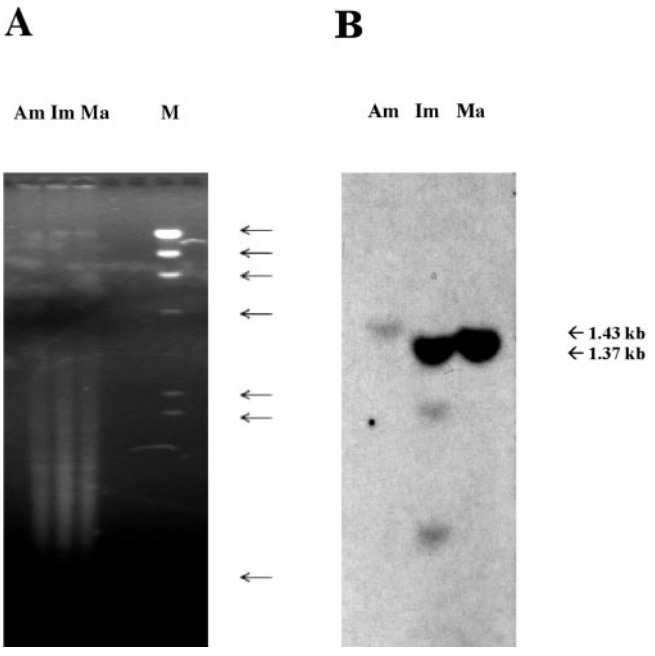

Figure 4. Amplification of *SvMT2b* sequences in copper-tolerant populations. A, Electrophoresis of the *Mbo*I-digested DNA (ethidium bromide-stained gel; each lane was loaded with 6 μg total leaf DNA of a single plant, digested with *Mbo*I). Arrows indicate the sizes of the marker fragments (DNA molecular-weight marker II, DIG-labeled [Boehringer Mannheim]; from the top, 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kb, respectively). B, Southern hybridization using a DIG-labeled *SvMT2b* cDNA probe. Sizes of the fragments were estimated by reference to the sizes of the marker. Am, Amsterdam; Im, Imsbach; Ma, Marsberg; M, Marker.

expression was largely unaffected by copper treatment (Fig. 3) and the level of *SvMT2b* expression was higher in leaves than in roots (data not shown).

Southern-blot analysis showed considerable differences in band intensities between the populations (Fig. 4B), whereas DNA loading was similar for all the samples (Fig. 4A). Digestion of leaf DNA with *Mbo*I and hybridization with *SvMT2b* probe generated a faint band at 1.43 kb for Amsterdam and a very intense band at 1.37 kb for Imsbach and Marsberg. Two smaller faint bands were also detected for Imsbach DNA.

SvMT2b mRNA Expression Analysis in Interecotypic Crosses

Two pair crosses were made between plants from the highly copper-tolerant population Imsbach ($EC_{100} > 400$ μM) and the copper-sensitive population Amsterdam ($EC_{100} < 12$ μM). Thirty F_1 plants were pair-crossed to produce F_2 seed. These seeds were pooled and approximately 500 F_2 seedlings were screened for copper tolerance (see "Materials and Methods"), using the lowest copper concentration that completely stopped root growth as a tolerance measure (Schat and Ten Bookum, 1992b). Eighteen seedlings with the sensitive Amsterdam phenotype ($EC_{100} \leq 12$ μM) and 12 copper-tolerant seedlings ($EC_{100} \geq 100$ μM) were selected and pair-crossed (sensitive $\times$ sensitive and tolerant $\times$ tolerant) to produce nine sensitive and nine tolerant F_3 families. Twenty-four plants of each of these families were screened for tolerance. The copper-sensitive F_3 families appeared to be devoid of any copper-tolerant individuals. The tolerant F_3 families showed a degree of segregation (EC_{100} values between 50 and 400 μM $CuSO_4$) but were devoid of any copper-sensitive individuals. The average tolerance levels in these families (EC_{100} between 100 and 200 μM) were consistently lower than that of the tolerant parent population Imsbach (EC_{100} approximately 500 μM).

The expression levels of the *SvMT2b* gene in copper-sensitive and copper-tolerant F_3 plants were determined to investigate the role of this gene in copper tolerance in *S. vulgaris*. It seemed that the copper-sensitive F_3 plants (1–9) varied discretely in *SvMT2b* expression (Fig. 5A). The plants from the families 3, 4, and 8 had a low expression level comparable with that of Amsterdam plants. The copper tolerant F_3 plants (10–18) showed the same variation in *SvMT2b* mRNA levels. The plants from the families 10 and 17 showed low expression.

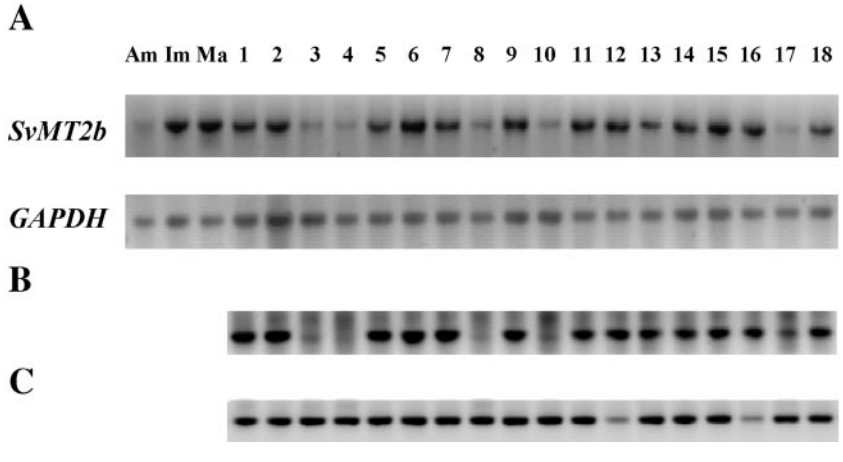

Figure 5. SvMT2b expression in copper-sensitive (1–9) and copper-tolerant (10–18) F_3 plants. A, Quantitative RT-PCR products of *SvMT2b* in root RNA (the plants were grown on 0.1 μM $CuSO_4$; a quantitative RT-PCR of *GAPDH* was used as an internal control). Allele-specific PCR products of *SvMT2b* obtained with Imsbach-specific primers (B) and with Amsterdam-specific primers (C). Am, Amsterdam; Im, Imsbach; Ma, Marsberg.

There was a perfect correlation between high *SvMt2b* expression, as established with quantitative RT-PCR, and the presence of at least one Imsbach allele, as established by allele-specific PCR. Imsbach allele-specific primers did not produce clear bands for the plants of the families with low *SvMT2b* expression, i.e. 3, 4, 8, 10, and 17, showing that these were homozygous for the Amsterdam allele (Fig. 5B). Only plants from the tolerant families 12 and 16 were homozygous for the Imsbach allele (Fig. 5C).

Enhanced *SvMT2b* expression was more or less evenly distributed over the sensitive and the tolerant F_3 selection lines, implying that this gene does not act as a primary determinant in copper tolerance. The possibility remains that it would act as a hypostatic enhancer of the level of tolerance in tolerant plants, however. This was investigated by genotyping less tolerant ($EC_{100} < 100$ μM $CuSO_4$) and more tolerant ($EC_{100} > 150$ μM $CuSO_4$) plants selected from tolerant F_4 families that segregated for *SvMT2b* expression. These F_4 families were produced by paircrossing eight tolerant F_3 plants with different genotypes for *SvMT2b* (four crosses between a heterozygote and an Amsterdam-type homozygote, and one heterozygote × heterozygote cross) but equal levels of tolerance. The resulting five F_4 families were screened for tolerance (25 plants per family) and the five least tolerant, as well as the five most tolerant plants of each of the families were genotyped for *SvMT2b* (see below). High-level tolerance appeared to be significantly positively associated with the possession of the highly expressed Imsbach allele (G-test with Yates' correction: $G = 21.7$; $P < 0.001$). Plants lacking the Imsbach allele were over-represented among the less tolerant plants (expected 43%, observed 90% [$n = 20$]), whereas plants possessing the Imsbach allele were over-represented among the more tolerant ones (expected 57%, observed 85% [$n = 20$]) (Fig. 6).

Pair crosses were also made between highly copper-tolerant plants from the population Imsbach and the moderately copper-tolerant plants from the population Marsberg. Three F_1 plants derived from different crosses were selfed, and the F_2 families were screened for tolerance. Six of the least tolerant (EC_{100}: 50–100 μM $CuSO_4$) and six of the most tolerant ($EC_{100} > 250$ μM $CuSO_4$) F_2 seedlings of each F_2 family were genotyped for *SvMT2b*. There was no significant cosegregation of allele origin with copper tolerance in these crosses. Sixteen of 18 plants tested, both from the low-tolerance and high-tolerance selected lines, possessed one or two Imsbach alleles, which strongly suggests that the differential tolerance of Imsbach and Marsberg plants is unrelated to the difference in the primary structure of the predicted SvMT2b proteins.

DISCUSSION

The *SvMT2b* gene was classified as type 2 within the MT-II class due to the presence of Cys-Cys and Cys-×-×-Cys motifs in the N-terminal domain (Robinson et al., 1993). Already 27 genes of this type have been referred to by Rauser (1999). Transcripts of MT-IIs occur in various of plant organs such as roots, leaves, stems, flowers, and seeds, either constitutively, or induced by different environmental conditions (Rauser, 1999). *S. vulgaris MT2b* is highly homologous with *MT* genes found in *M. crystallinum* and in *Oryza sativa* (*OsMT-2*; Hsieh et al., 1996). Responses of *MT* expression to copper treatment are different between these species despite the high homology. In Arabidopsis the *MT2b* mRNAs were abundant in leaves and to a lesser extent in roots from mature plants and exposure of seedlings to copper resulted in only a slight increase (Zhou and Goldsbrough, 1995). This pattern is comparable with the results found in the present study. Mineta et al. (2000) found that *MT2a* promotor activity in copper-treated Arabidopsis was highest in and around the vascular tissue, whereas copper accumulated mainly in the cortex. Also, *MT2b* promotor activity was found to be highest in the stele (Bundithya and Goldsbrough, unpublished data; mentioned in Cobbett and Goldsbrough [2000]). These results suggest that copper itself is not directly involved in *MT2* transcription.

The Southern blot showed big differences in band intensities between Amsterdam, Marsberg, and Imsbach (Fig. 3b). Recent results have revealed that *SvMT2b* is composed of two exons and a long intron, inserted after bp 69 of the coding sequence (A. Tervahauta, unpublished data). The *SvMT2b* coding sequence contains one *Mbo*I restriction site located in exon I. The probe that we used was specific to exon II, implying that the number of *SvMT2b* containing restriction fragments should be equal to the number of copies of this gene in the genome. The probe, which completely matched exon II from Imsbach

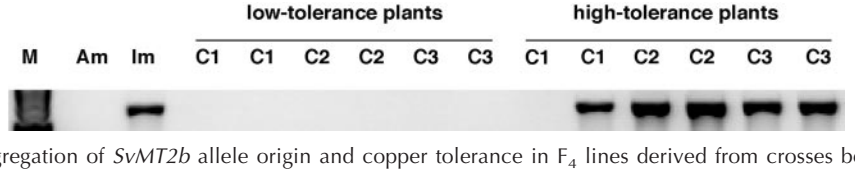

Figure 6. Cosegregation of *SvMT2b* allele origin and copper tolerance in F_4 lines derived from crosses between copper-tolerant F_3 plants. PCR products of *SvMT2b* using Imsbach allele-specific primers and leaf DNA from plants of the *S. vulgaris* populations Amsterdam and Imsbach, and from 12 plants of different F_4 lines (C1, C2, and C3). Low-tolerance plants and high-tolerance plants were selected from crosses between tolerant low-expression homozygotes and tolerant high-expression heterozygotes. M, Marker (250-bp DNA mass ladder, MRC Holland); Am, Amsterdam; Im, Imsbach.

plants, contained 5- and 4-bp mismatches with exon II from Amsterdam and Marsberg plants, respectively, which is far from sufficient to produce the band intensity differences under the stringency conditions applied. Thus, it seems that the intense bands in Imsbach and Marsberg must have resulted from the presence of a number of identical *SvMT2b* containing repeats. The size of these repeats is unknown, but may be longer than 1.37 kb, because the intron, which has not been completely sequenced, contains at least two *Mbo*I sites (A. Tervahauta, unpublished data). Further analysis of the repeat structure by long PCR and reverse PCR has been unsuccessful thus far. Tandem repeat of *SvMT2b* seems to be likely however, and might account for the overexpression in the tolerant plants. To our knowledge, no other plant MT tandem repeat has been described so far. However, a tandem repeat of the yeast MT gene, *CUP1*, has been found in cadmium-resistant (Tohoyama et al., 1996) and copper-resistant strains (Fogel et al., 1983; Tohoyama et al., 1992).

High *SvMT2b* mRNA accumulation in plants from the Amsterdam × Imsbach F_3 crosses was strictly dependent on the presence of at least one Imsbach allele. Low-expression F_3 plants were all homozygous for the Amsterdam allele. This implies that high expression could be conferred by a dominant cis-acting regulatory component or, possibly, a closely linked trans-acting component. Enhanced *SvMT2b* expression as such is not sufficient to produce increased copper tolerance, relative to the sensitive parent population, as indicated by the presence of high-expression plants among the non-segregating copper-sensitive F_3 families. However, a strict cosegregation between high-level copper tolerance and *SvMT2b* overexpression did occur in tolerant F_4 families (Fig. 5). Thus, overexpression of *SvMT2b* can confer additional copper tolerance, although only in combination with components of the genetic background of a copper tolerant plant, suggesting that *SvMT2b* acts as a hypostatic enhancer, rather than as a primary tolerance gene. An important role for hypostatic enhancers in copper tolerance has also been demonstrated in *Mimulus guttatus* (Macnair, 1983; Smith and Macnair, 1998).

There are differences in the predicted amino acid sequences of the SvMT2b protein between the *S. vulgaris* populations. This could result in a change in the three dimensional structure of the protein (Keeton et al., 1993). It is difficult to assess whether such changes have any influence on the stability or the physiological role of the protein in the plant. At any rate, the differences in SvMT2b amino acid sequences between Imsbach and Marsberg had no effect on the copper tolerance as shown by the lack of cosegregation with tolerance in the F_2 families derived from Imsbach × Marsberg crosses.

Overexpression of *SvMT2b* restored cadmium and copper tolerance in hypersensitive yeast, suggesting that the SvMT2b protein increased the cellular sequestration capacity, probably through binding the metals. We were unable to assess the cellular SvMT2b protein levels in *S. vulgaris*, probably because of their sensitivity to oxidation. However, the highly significant cosegregation of enhanced *SvMT2b* expression with high tolerance in tolerant F_4 families indicates that the mRNAs were indeed translated into functional proteins. It is very difficult to detect this type of MT proteins, therefore not much is known about post-transcriptional processing of MTs. However, Murphy et al. (1997) succeeded in detecting MT proteins in Arabidopsis, and they found no major discrepancies between the patterns of mRNA expression and the corresponding protein levels.

SvMT2b overexpression in both the Imsbach and Marsberg populations must have resulted from independent evolution. Gene flow between these populations is very unlikely (Schat et al., 1996). Moreover, the Marsberg *SvMT2b* sequence is much more similar to the Amsterdam sequence than to the Imsbach one. Thus, *MT2b* amplification might be a common phenomenon. Unequal crossing-over constitutes a plausible mechanism for such gene amplifications. The absence of high *SvMT2b* expression in the populations Amsterdam, Wijlre, and Gaschurn suggests that there may be selection against it on non-metalliferous soil.

In conclusion, the copper-tolerant populations showed a constitutively higher *SvMT2b* expression, which could result from gene amplification. This overexpression does not produce copper tolerance by itself but merely increases the level of tolerance produced by one or more epistatic primary tolerance genes. Further studies of copper tolerance genes and their interactions are necessary to get a better understanding of the different mechanisms involved in copper tolerance in *S. vulgaris*.

MATERIALS AND METHODS

Plant Materials

Seeds of *Silene vulgaris* plants were collected from the copper mines near Imsbach and Marsberg (Germany) and from the botanical garden of the Vrije Universiteit of Amsterdam (The Netherlands). Characteristics of these sites and of the local *S. vulgaris* populations have been given in Schat and Ten Bookum (1992a). In addition, seeds were also collected from non-metallicolous populations growing in limestone grassland at Wijlre (The Netherlands) and a subalpine meadow near Gaschurn (Austria).

Tolerance Testing

Seed germination, hydroponic preculture, nutrient and test solution compositions, and growth chamber conditions were exactly as in Schat et al. (1996). Tolerance testing was performed using a sequential exposure method described in Schat and Ten Bookum (1992b). In short, plants were

exposed to a sequence of linearly increasing concentrations of copper in the test solution (2-d exposure to each concentration). Prior to exposure to the first concentration, the roots were stained black by dipping them in a stirred suspension of finely powdered active carbon (Schat and Ten Bookum, 1992b). At each transfer to a higher concentration the plants were checked for root growth and subsequently restained. The lowest copper concentration that completely stopped root growth (lowest 100%-effect-concentration [EC_{100}]) was taken as a tolerance measure. The sensitive parent population (Amsterdam) was screened using a 4-, 8-, 12-, and 16-μM $CuSO_4$ concentration series. The tolerant populations were screened using 25-μM (Marsberg), and 100-μM (Imsbach) concentration steps, respectively. To select sensitive and tolerant plants from the Amsterdam $\times$ Imsbach F_2 crosses, 250 seedlings were tested using 4-μM concentration steps and another 250 using 50-μM steps. Supposedly sensitive and tolerant F_3 lines derived from crosses among sensitive ($EC_{100} < 12$ μM) and tolerant ($EC_{100} > 100$ μM) F_2 plants were screened using 4-μM and 50-μM intervals, respectively. Moderately tolerant and highly tolerant plants from Marsberg $\times$ Imsbach F_2 crosses were selected using 25- and 50-μM concentration steps, respectively. Tolerant F_4 families segregating for MT2b expression were screened with 50-μM intervals.

cDNA Library Construction and Screening

A λgt11 cDNA library was prepared from leaves of untreated copper-tolerant plants (population Imsbach). Total RNA was isolated according to the guanidine hydrochloride method (Logemann et al., 1987). Poly-A-mRNA was isolated using an Oligotex mRNA kit (Qiagen USA, Valencia, CA). cDNA was prepared with a cDNA Synthesis Kit (Pharmacia Biotech, Piscataway, NJ), size-selected with a SizeSep 300 Spun Column (Pharmacia Biotech), and ligated with *Eco*RI-adaptors and finally with *Eco*RI-precut λgt11-arms. The library was packaged using a Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA), titrated (approximately 1×10^6 plaques), and amplified (5×10^{11} pfu/mL). The amplified library was plated (approximately 6,000 plaques on a plate of 9 cm in diameter) and lifted on Nylon membranes (Boehringer Mannheim/Roche, Basel). DIG-labeled DNA probes were produced using specific primers for Arabidopsis MT1a, MT2a, MT2b, and MT3 (MT1a, 5′-GAATTCGGCACGAGGAAGAA-3′ and 5′-AGTTGTCGCACTCCTTGTTG-3′; MT2a, 5′-CCAGAATTCTCGAGAAAAATGTCTTGC-3′ and 5′-GTCGAATTCACTTGCAGGTGCAAG-3′; MT2b, 5′-TGTCTTGCTGTGGTGGAA-3′ and 5′-TCATTTGCAGGTACAAGGGTT-3′; MT3, 5′-ATGTCAAGCAACTGCGGAAGC-3′ and 5′-AAGTGCAGTTGACGCAGCTGCAA-3′) and plasmids containing the corresponding Arabidopsis cDNAs as a template (Zhou and Goldsbrough, 1995). Hybridization was performed according to the manufacturer's instructions, and hybridizing plaques were replated. After a second hybridization, positive plaques were taken in liquid lysis. Lambda-lysates were boiled for 5 min, centrifuged, and 2 μL was taken as a sample in PCR using λgt11 specific primers: 5′-GGACGTCGACGGTGGCGACGACTCCTGGAG-3′ and 5′-CGCCGCGGCCGCACCAACTGGTAATGGTAGCG-3′. PCR products were sequenced (ALFexpress, Pharmacia Biotech) using fluorescein isothiocyanate-labeled λgt11-specific sequencing primers.

Characterization of *SvMT2b* Expression Patterns

Plant Culture

The plants were grown hydroponically for 2 weeks before DNA and/or RNA extraction. Some of the plants were exposed to 50 μM $CuSO_4$ for 24 or 48 h before harvest. At harvest, roots were shortly rinsed with distilled water and the whole root system or/and a mature leaf was cut off, immediately frozen in liquid nitrogen, and stored at −80°C until extraction.

DNA/RNA Isolation

DNA isolation was performed according Doyle and Doyle (1990). Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen).

cDNA Preparation

Single-stranded cDNA was prepared from 2 μg of RNA, 20 pmol of RACE dT primer, 0.25 mM deoxynucleoside triphosphates (Life Technologies/Gibco-BRL, Cleveland), 200 units of M-MLV reverse transcriptase (Life Technologies/Gibco-BRL), and 1 $\times$ RT first strand buffer (Life Technologies/Gibco-BRL), in a total volume of 20 μL.

Sequencing of SvMT2b Alleles

The primers 5′-TTTCAGTAATTTAATCAGCG-3′ and 5′-GCTTGTTTTACCCTGTTGAG-3′, based on the non-coding regions of a *MT2b*-like cDNA (*SvMT2b*) found in the library (see above), were used to amplify and sequence the coding regions of the corresponding cDNAs of plants from the populations Amsterdam and Marsberg. PCR was performed using 2 μL of cDNA, 20 pmol of each primer, 1 mM deoxynucleoside triphosphates (0.25 m each) (Life Technologies/Gibco-BRL), 1 unit of *Taq* polymerase (MRC Holland), and 1 $\times$ *Taq* buffer (MRC Holland), adjusted to a total volume of 25 μL with sterilized water. An annealing temperature of 55°C and 34 cycles were used. PCR products of the expected lengths were sequenced. Sequencing was performed using the Terminator Cycle Sequencing Core Kit (Perkin-Elmer Applied Biosystems, Foster City, CA).

Allele-Specific PCR

The primers used for the allele-specific PCR were 5′-GAAAATGTCGTGCTGTAATGGA-3′ and 5′-GTTCATTTGCAAGTGCAAGGG-3′ for the Imsbach allele and 5′-GAAAATGTCGTGCTGTAATGGT-3′ and 5′-GCGTCGAAGTATGAGGCCTT-3′ for the Amsterdam allele, giving

SvMT2b PCR products of 243 and 159 bp, respectively. The allele-specific PCR was performed using 2 μL of cDNA or 100 ng of total genomic DNA, 20 pmol of each primer, 1 mM deoxynucleoside triphosphates (0.25 mM each) (Life Technologies/Gibco-BRL, Cleveland), 1 unit of *Taq* polymerase (MRC Holland), and 1 × *Taq* buffer (MRC Holland), adjusted to a total volume of 25 μL with sterilized water, in a PCR program with 34 cycles and an annealing temperature of 69°C (2 min 94°C; 34 cycles of [30 s 94°C, 45 s 69°C, 1 min 30 s 72°C]; 5 min 72°C; storage at 4°C) (Biometra Tgradient). Eight microliters of each PCR product was run in an agarose gel with a 250-bp DNA mass ladder (MRC Holland) and photographed in UV-light.

Quantitative RT-PCR

The primers used for the quantitative RT-PCR were 5′-GAAAATGTCGTGCTGTAATGG-3′ and 5′-AAGGGTTGCACTGGCAGTTG-3′, based on a part of the *SvMT2b* cDNA that was identical in plants from Amsterdam, Marsberg, and Imsbach. The house-keeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as a positive internal control. The primers 5′-TGGGATCCTCACTGACAAGGACAAGGCT-3′ and 5′-TGAATTCCCCCATTCGTTGTCGTACC-3′, designed on the basis of published sequences of *GAPDH* genes from other plant species (GenBank Accession no. X75597; Niu et al., 1994; GenBank Accession no. X07156; Brinkmann et al., 1987), were used to identify *S. vulgaris GAPDH.* PCR and sequencing of a part of the *GAPDH* gene, using cDNA of plants from Imsbach as a template, was performed as described in "Sequencing of SvMT2b alleles" (see above). The *S. vulgaris GAPDH* primers used for the quantitative RT/PCR were 5′-GTTATCATCTCAGCTCCTAG-3′ and 5′-GCAACACCTTTCCAACAGCCT-3′. Quantitative PCR was performed using 4 μL of cDNA, 20 pmol of each primer, 1 mM deoxynucleoside triphosphates (0.25 mM each) (Life Technologies/Gibco-BRL), 1 unit of *Taq* polymerase (MRC Holland), and 1 × *Taq* buffer (MRC Holland), adjusted to a total volume of 25 μL with sterilized water. A PCR program of 15 cycles and an annealing temperature of 55°C was used (2 min 94°C; 15 cycles of [30 s 94°C, 45 s 55°C, 1 min 30 s 72°C]; 5 min 72°C; storage at 4°C) (Biometra Tgradient). This gives *SvMT2b* and *GAPDH* PCR products of 227 and 313 bp, respectively. Twelve microliters of each PCR product was electrophoresed in an agarose gel and photographed in UV light.

Northern-Blot Analysis

Eight micrograms of total RNA isolated from roots of untreated and copper-treated (50 μM $CuSO_4$ for 0, 24, and 48 h) plants from the populations Amsterdam and Imsbach were used. Preparations of samples, formaldehyde agarose gel electrophoresis, and blotting on nylon membrane was performed according to Sambrook et al. (1989). The primers used to make the radioactive cDNA probe were 5′-GAAAATGTCGTGCTGTAATGG-3′ and 5′-CATTTGCAAGTGCAAGGGT-3′ and Imsbach cDNA was used as a template, resulting in a probe of 240 bp. Labeling of the radioactive probe (isotope 32^P), overnight hybridization at 65°C and washings (brief rinsing at 65°C in 2× SSC and washing for 10 min in 0.5× SSC/0.1% [w/v] SDS at 65°C) were performed according to Church and Gilbert (1984). The blot was scanned with a phosphor imager.

Southern-Blot Analysis

Six micrograms of total DNA, isolated from leaves of plants from the populations Amsterdam, Marsberg, and Imsbach, was digested overnight at 37°C with the restriction enzyme *Mbo*I and electrophoresed overnight on a 0.7% agarose gel in 0.5 × TBE buffer with a DIG-labeled DNA marker II (Boehringer Mannheim). DNA was blotted on a Nylon membrane (Boehringer Mannheim). The primers used to make the cDNA probe were 5′-CAAGATGTTCCCTGAGTTT-3′ and 5′-CATTTGCAAGTGCAAGGGT-3′ and Imsbach cDNA was used as a template, resulting in a probe of 168-bp (part of the gene without an intron). DIG labeling of the probe, hybridization, and washings were performed as described in the Boehringer Mannheim manual (GmbH DIG system; 1995). Hybridization occurred overnight at 42°C. The blots were washed twice in 2× SSC/0.1% (w/v) SDS at room temperature for 15 min and two times more in 0.2× SSC/0.1% (w/v) SDS at 68°C for 15 min. The blots were exposed to a chemiluminescence film (Hyper film ECL, Amersham Life Science). DNA loading was checked by ethidium bromide staining.

Complementation of Copper-Sensitive and Cadmium-Sensitive Yeast Mutants

SvMT2b was amplified with PCR from a λgt11 clone lysate using primers 5′-GCGGAATTCGATGTCGTGCTGTAATGGAA-3′ and 5′-CGGCTCGAGCTCATTTGCAAGTGCAAGGG-3′ containing *Eco*RI and *Xho*I restriction sites for cloning into the pAJ401 *Escherichia coli* (yeast shuttle vector). pAJ401 was derived from pFL60 (Minet and Lacroute, 1990) by interchanging *Eco*RI and *Xho*I cloning sites. Recombinants were first introduced into *E. coli*, selected by ampicillin resistance, and the presence of *SvMT2b* gene was confirmed with PCR and sequencing using vector specific primer 5′-CATCAAGGAAGTAATTATCT-3′. Plasmid DNA miniprep was made and introduced into cadmium-sensitive (JWY53, Δ*ycf1*, Wemmie et al., 1994) and copper-sensitive yeasts (DBY746, a common laboratory yeast strain; DM771-6C, Δ*cup1*). First transformant selection in yeast was made by uracyl auxotrophy.

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